BH3 Mimetic ABT-737 and a Proteasome Inhibitor Synergistically Kill Melanomas through Noxa-Dependent Apoptosis

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The Bcl-2 family is important in modulating sensitivity to anticancer drugs in many cancers, including melanomas. The BH3 mimetic ABT-737 is a potent small molecule inhibitor of the anti-apoptotic proteins Bcl-2/Bcl-X_L/Bcl-w. In this report, we examined whether ABT-737 is effective in killing melanoma cells in combination with the proteasome inhibitor MG-132, and further evaluated the mechanisms of action. Viability, morphological, and Annexin V apoptosis assays showed that ABT-737 alone exhibited little cytotoxicity, yet it displayed strong synergistic lethality when combined with MG-132. In addition, the detection of Bax/Bak activation indicated that the combination treatment synergistically induced mitochondria-mediated apoptosis. Furthermore, mechanistic analysis revealed that this combination treatment induced expression of the pro-apoptotic protein Noxa- and caspase-dependent degradation of the anti-apoptotic protein, Mcl-1. Finally, siRNA-mediated inhibition of Mcl-1 expression significantly increased sensitivity to ABT-737 in these cells, and knocking down Noxa expression protected the cells from cytotoxicity induced by the combination treatment. These findings demonstrate that ABT-737 combined with MG-132 synergistically induced Noxa-dependent mitochondrial-mediated apoptosis. In summary, this study indicates promising therapeutic potential of targeting anti-apoptotic Bcl-2 family members in treating melanoma, and it validates rational molecular approaches that target anti-apoptotic defenses when developing cancer treatments.

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INTRODUCTION

Malignant melanoma is largely unresponsive to existing therapies and has a very poor prognosis (Buzaid, 2004; Cummins *et al.*, 2006; Gogas *et al.*, 2007). In addition, there has been little progress over the past 30 years in medical treatment of this disease. Thus, the paucity of effective treatments for malignant melanoma is a pressing issue in medicine.

The recent use of proteasome inhibition in clinical practice to induce apoptosis in cancer has produced interesting results. However, proteasome inhibition alone is neither complete nor sustained because of the need to limit secondary toxicity (Adams, 2004). In addition, pharmacokinetic analyses have indicated that proteasome inhibitors are rapidly inactivated by hepatic detoxification (Schwartz and Davidson, 2004). Therefore, compounds that synergize with proteasome inhibitors to lower required dosages and/or to minimize the time delay from proteasome inhibition to the activation of the apoptotic machinery could have important clinical implications.

Apoptosis is one of the main pathways contributing to the cytotoxic effects of conventional cancer treatments, and functional apoptotic pathways are required for the success of most treatments. During melanoma's progression, malignant cells become 'bullet proof' against a variety of chemotherapeutic drugs by exploiting their intrinsic apoptotic defenses and by reprogramming their proliferation and survival pathways (Soengas and Lowe, 2003).

The Bcl-2 family of proteins is crucial in regulating apoptosis (see review Thomadaki *et al.*, 2006), especially mitochondria-dependent apoptotic cell death. This family can be divided into three groups: anti-apoptotic proteins, including proteins such as Bcl-2, Bcl-X_L, Bcl-w, and Mcl-1; multi-domain pro-apoptotic proteins Bax and Bak; and pro-apoptotic BH3-only proteins, including Noxa, Bim, Bid, Bad, Bmf, and Bik.

Molecular-targeted therapy holds the promise of providing new and more effective treatment options with minimal toxicity (Weinstein and Joe, 2006). Compounds have been developed to target activated kinases or receptors (Weinstein and Joe, 2006), but there are also many advantages for

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Abbreviations: ANOVA, analysis of variance; EB/AO, ethidium bromide and acridine orange; MTS, a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2 (4-sulfophenyl)-2H-tetrazolium], inner salt; SiRNA, short interfering RNA

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targeting the defective apoptotic machinery in cancer cells: (1) Even though different gene mutations exist in different types of cancers, downstream pathways that include anti-apoptotic defenses are very similar. (2) Abnormally regulated apoptotic signaling is one of the hallmarks of cancer. (3) Finding molecular targets in anti-apoptotic defenses will provide molecular targets for cancer therapy that differ from targeting kinases or receptors.

ABT-737 (developed by Abbott Laboratories) is a potent small molecule inhibitor of the anti-apoptotic proteins Bcl-2, Bcl-X_L, and Bcl-w with an affinity 2–3 orders of magnitude more potent than any previously reported compounds (Letai, 2005; Oltersdorf *et al.*, 2005). It acts like a BH3—only protein to antagonize anti-apoptotic Bcl-2 family members, thereby diminishing their ability to inhibit apoptosis (Oltersdorf *et al.*, 2005). Many teams have recently reported the high efficacy of ABT-737 either as a single agent or as a chemo-potentiator in combination with other chemotherapeutic agents to treat multiple types of cancers, including lymphoma, small-cell lung carcinoma, various leukemias, etc. (Adams *et al.*, 2005; Oltersdorf *et al.*, 2005; Certo *et al.*, 2006; Konopleva *et al.*, 2006; Shoemaker *et al.*, 2006; van Delft *et al.*, 2006; Chauhan *et al.*, 2007; Chen *et al.*, 2007a, b; Kang *et al.*, 2007; Kohl *et al.*, 2007).

In this study, we explored whether ABT-737 is effective in treating human melanoma cell lines and whether it potentiates killing of melanoma cells by the proteasome inhibitor, MG-132. We found that the two drugs acted synergistically to activate Noxa-dependent mitochondrial-mediated apoptosis. Mechanistic analysis suggests that this combination increases pro-apoptotic protein Noxa expression, neutralizes all main anti-apoptotic Bcl-2 proteins, and induces accelerated Noxa-mediated synergistic killing.

RESULTS

ABT-737 synergistically killed human melanoma cells when combined with MG-132

There are important clinical implications for finding compounds that synergize with proteasome inhibitors to lower the required doses and/or to minimize the time delay from proteasome inhibition to the activation of apoptotic machinery. We examined the effects of ABT-737 alone or in combination with MG-132 in treating A375, WM852c and HS294T human melanoma cells using a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2 (4-sulfophenyl)-2H-tetrazolium], inner salt (MTS) assays and ethidium bromide and acridine orange (EB/AO) staining. The relative cell viability present in Figure 1 reveals that ABT-737 alone caused little cytotoxicity in these cells, but both 1.1 and 3.3 μM ABT-737 significantly increased killing effects of MG-132. To quantify whether these combinations had synergistic, additive or antagonistic effects, we used Calcusyn software to calculate the combination index (CI) value for each experimental combination with the data from Figure 1 (Figure S1). We found that the MG-132 and ABT-737 combination treatments displayed strong synergistic killing for these melanoma cell lines, and similar results were observed with EB/AO staining (Figure S2).

ABT-737 combined with MG-132 induced apoptosis synergistically in human melanoma cell lines through the intrinsic pathway (mitochondria mediated)

We performed additional assays to further examine whether the combination of ABT-737 and MG-132 induces apoptosis synergistically in melanoma cells (Figure 2).

First, Figure 2a illustrates bright field microscopic morphology of A375 and WM852c melanoma cells. Compared with the DMSO control, ABT-737 alone did not affect the cellular morphology significantly, and MG-132 alone minimally increased the number of rounded cells, which still appeared alive. In contrast, the combination treatment caused almost all cells to detach and appear dead.

Second, Figure 2b presents the results from Annexin V staining of A375 and WM852c melanoma cells. The combination of ABT-737 and MG-132 induced many more apoptotic cells than the sum total of apoptotic cells induced by either drug alone, demonstrating the synergistic killing effects of the combination. Statistical analysis indicated that only the combination treatment increased apoptosis significantly from DMSO controls in both cell lines.

Third, to further delineate the pathway, we examined whether these treatments induced Bax/Bak activation (Figure 2c). Bax and Bak are multi-domain pro-apoptotic Bcl-2 family members that are essential for mitochondria-dependent apoptosis (Wei *et al.*, 2001). Bax and/or Bak activation is associated with conformational changes that can be detected by specific antibodies, and it is a good indicator of mitochondria-mediated apoptosis (Griffiths *et al.*, 1999; Nechushtan *et al.*, 1999). ABT-737 or MG-132 alone induced activation of Bax/Bak in a small percentage of WM852c melanoma cells, but the number of Bak/Bax-activated cells induced by the combination treatment was more than double the amount of the sum total of positive cells induced by ABT-737 and MG-132 alone, indicating strong synergism as opposed to additive effects (Figure 2c). Statistical analysis indicated that only the combination treatment increased active Bak/Bax positive cells significantly from DMSO controls. Similar results were seen with A375 cells (Figure 2c).

Taken together, the synergistic induction of mitochondria-mediated apoptosis by ABT-737 and MG-132 was demonstrated by morphology of the treated cells, measurements of the apoptotic cell populations by Annexin V/propidium iodide staining, and the detection of Bax/Bak activation.

Combination of ABT-737 and MG-132 induced expression of the pro-apoptotic protein Noxa- and caspase-dependent degradation of the anti-apoptotic protein Mcl-1

MG-132 has been reported to induce expression of the pro-apoptotic protein Noxa, which has been shown to activate apoptosis through antagonizing Mcl-1 function in melanoma (Fernandez *et al.*, 2005; Qin *et al.*, 2005). Thus, we hypothesized that ABT-737 would block the function of Bcl-2, Bcl-X_L, Bcl-w, whereas MG-132 would induce Noxa production, neutralizing the anti-apoptotic function of Mcl-1. Thus, ABT-737 and MG-132 together would inhibit all four anti-apoptotic proteins to synergistically induce apoptosis.

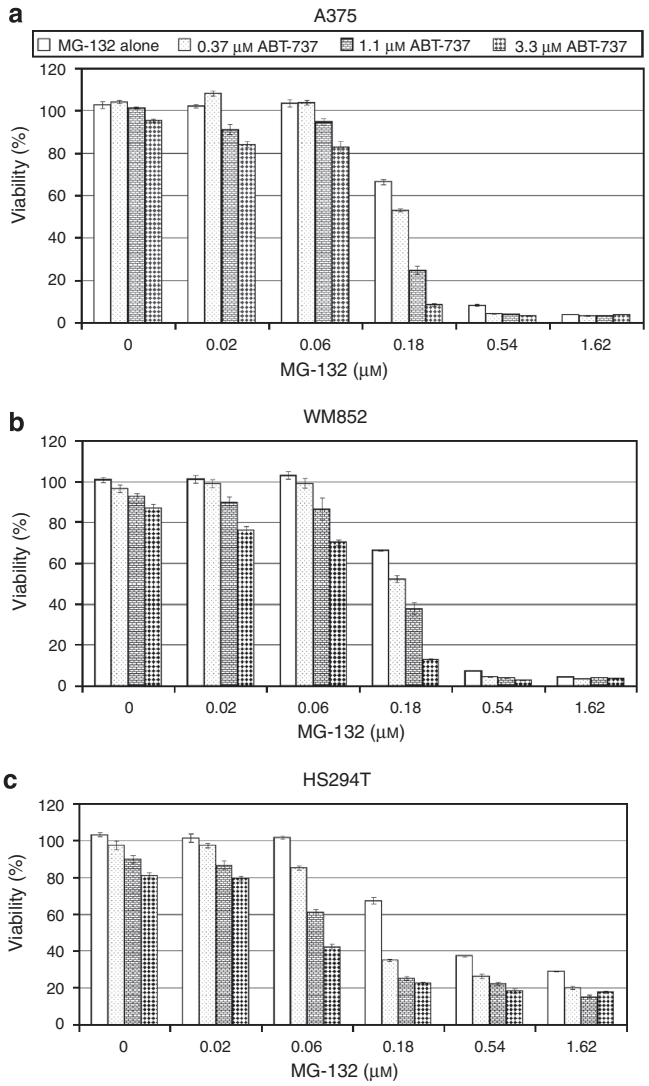

Figure 1. Combination of ABT-737 and MG-132 killed human melanoma cells synergistically. Cells were seeded in 96-well plates 24 hours before treatment with ABT-737, MG-132, or both drugs at indicated doses. MTS assays were performed 48 hours post-treatment. Averaged media controls for multiple plates were set as 100% viability; (**a**) A375 cells, (**b**) WM852c cells, and (**c**) HS294T cells. Independent experiments have been repeated at least three times, and similar results have been observed.

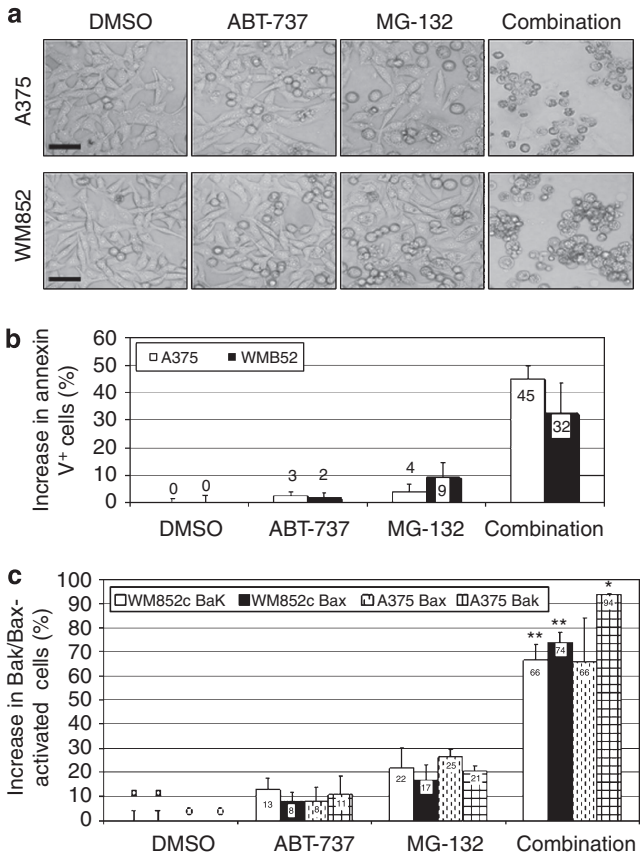

Figure 2. Combination of ABT-737 and MG-132 induced mitochondrial-mediated apoptosis in melanoma cells synergistically. A375 and WM852c melanoma cells were treated for 24 hours with DMSO control, 1.1 μM ABT-737 and 0.18 μM MG-132, either alone or in combination. Percentage of apoptotic cells were analyzed using Annexin V staining. (**a**) Bright Field microscopy images of A375 and WM852c cells before being detached for staining. Scale bar = 100 μM. (**b**) Average increase ± SEM in apoptosis for treated A375 and WM852c cells. One-way ANOVA demonstrated significant differences among Annexin V^+ cells for all treatments of A375 ($F = 55.9$, $F_{crit} = 4.07$, $P = 1 \times 10^{-5}$) and WM852c ($F = 5.5$, $F_{crit} = 4.07$, $P = 0.02$). However, Tukey's *post hoc* analysis for both cell lines indicated that only the combination treatment significantly increased percentages of Annexin V^+ cells compared with DMSO controls for both A375 cells (**$P < 0.01$) and WM852c cells (*$P < 0.05$). (**c**) Average increase ± SEM in Bak/Bax-activated cells for treated WM852c or A375 cells. One-way ANOVA demonstrated statistically significant differences among active Bak/Bax positive cells for all treatments (Bak: $F = 21.5$, $F_{crit} = 4.07$, $P = 3 \times 10^{-4}$; Bax: $F = 54.9$, $F_{crit} = 4.07$, $P = 1 \times 10^{-5}$), but only the combination treatment produced significantly higher active Bak and Bax positive cells than DMSO control cells (*$P < 0.05$ and **$P < 0.01$). Data summarized three independent experiments.

To investigate this hypothesis, we examined changes in Bcl-2 family protein expression by Western blot. Only Noxa and Mcl-1 protein levels had consistent and dramatic changes induced by either one drug alone and/or the combination 24 hours post-treatment (Figure 3a and Figure S3). ABT-737 alone had little effects on most proteins examined, and MG-132 alone induced both Noxa and Mcl-1 levels substantially (Figure 3a). The combination treatment increased Noxa expression to similar levels as MG-132 alone. However, surprisingly, Mcl-1 expression in cells treated with the drug combination was reduced to levels similar to cells treated with vehicle control or ABT-737 alone (Figure 3a).

To examine the unexpected decrease in Mcl-1 expression in cells treated with the drug combination compared with increased levels of Mcl-1 in cells treated with MG-132 alone, we performed time course experiments (WM852c in Figure 3b and A375 in Figure S4). Results showed that Mcl-1 protein expression was induced by the combination treatment for up to 6 hours, but it was reduced to endogenous levels at 24 hours. Caspase 3 was activated at 24 hours but not the earlier time points.

Downregulation of Mcl-1 at the protein level can be achieved through proteasomal degradation (Cuconati *et al.*, 2003; Nijhawan *et al.*, 2003; Willis *et al.*, 2005) or caspase-dependent specific cleavage (Clohessy *et al.*, 2004; Michels *et al.*, 2004). In our case, we hypothesized that the latter

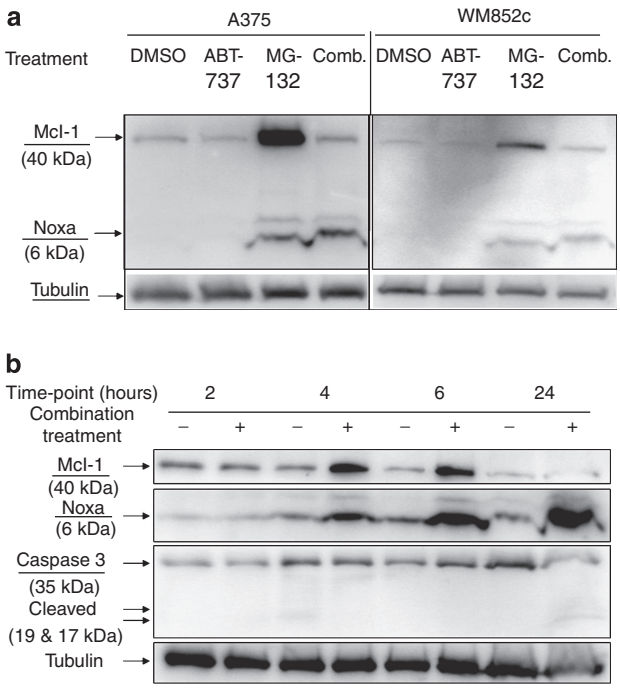

Figure 3. The effects of combination treatment with ABT-737 and MG-132 on Noxa and Mcl-1 protein expression. (**a**) Western blot: A375 or WM852c cells were treated in the same way as in Figure 2, and lysates were prepared 24 hours post-treatment before being subjected to western blot analysis. (**b**) Time course experiments of WM852c. Cells were treated with either vehicle control (DMSO) or the combination of 1.1 μM ABT-737 (ABT) and 0.18 μM MG-132 (MG), and lysates were prepared at indicated time points post-treatment before being subjected to western blot analysis.

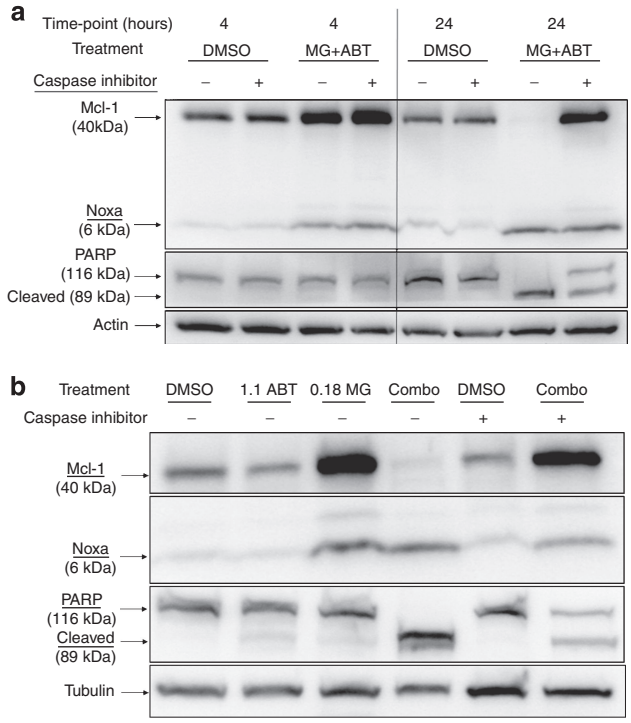

Figure 4. Caspase inhibitor blocked downregulation of Mcl-1 protein expression induced by ABT-737 and MG-132 combination treatment. (**a**) Western blots: A375 cells were treated with either vehicle control (DMSO) or the combination of 1.1 μM ABT-737 (ABT) and 0.18 μM MG-132 (MG). The general caspase inhibitor zVAD-fmk was used at 30 μM at the same time that other drugs were added. Lysates were prepared at either 4 or 24 hours post-treatment. (**b**) Western blot: A375 cells were treated with either DMSO, 1.1 μM ABT-737 (1.1 ABT) and 0.18 μM MG-132 (0.18 MG) or the combination of 1.1 μM ABT-737 and 0.18 μM (Combo). Thirty micromolar zVAD-fmk was added to the indicated cells at the same time of other treatments, and lysates were prepared 24 hours post-treatment.

scenario is more likely as MG-132 inhibits proteasome activity. To determine whether our observed Mcl-1 down-regulation was dependent on caspase activity, we employed the wide-spectrum general caspase inhibitor zVAD-fmk in our experiments.

Figure 4a shows that without zVAD-fmk, ABT-737 plus MG-132 induced Mcl-1 expression at 4 hours, but downregulated its expression back to endogenous levels at 24 hours, as we observed before. In contrast, at the 24 hours time point, zVAD-fmk dramatically increased Mcl-1 expression in cells treated with ABT-737 and MG-132 but not in the control-treated cells. However, the caspase inhibitor did not affect Noxa expression. Figure 4b also illustrates that at 24 hours, the caspase inhibitor dramatically increased full length Mcl-1 expression in the combination-treated cells to a level similar to cells treated with MG-132 alone in A375 cells. Similar results have been observed in WM852c cells (Figure S5).

Thus, these data suggest that the combination treatment induced pro-apoptotic protein Noxa expression, and caspase-dependent anti-apoptotic protein Mcl-1 degradation.

Knocking down Mcl-1 expression increased melanoma's sensitivity to ABT-737 and knocking down Noxa expression protected cells from killing induced by the combination of ABT-737 and MG-132

As pro-apoptotic Noxa mainly binds to the anti-apoptotic protein Mcl-1 but not Bcl-2, Bcl-X_L, or Bcl-w, the major function of Noxa in regulating apoptosis is to antagonize Mcl-1 (Chen *et al.*, 2005; Willis *et al.*, 2005). To further investigate the biological relevance of our observed Mcl-1 cleavage and Noxa induction, we examined whether knocking down Mcl-1 expression affected sensitivity to ABT-737 in melanoma cells using RNA interference (Figure 5). Inhibiting Mcl-1 expression by siMcl-1 alone was not sufficient to induce cell death. However, when the cells were treated with ABT-737, blocking Mcl-1 expression (siMcl-1) significantly increased Annexin V^+ cells, more than fivefold in both WM852c and A375 cells (Figure 5a). Immunoblot analysis indicated that siMcl-1 inhibited Mcl-1 expression by ~70% in both control or ABT-737 treated cells (Figure 5b). These results demonstrate that Mcl-1 mediates resistance to ABT-737, and this suggests that in the combination treatment, MG-132 potentiates ABT-737's ability to induce cell death by upregulating Noxa expression to antagonize Mcl-1.

We then investigated whether inhibiting Noxa expression affected the synergistic killing induced by the combination drug treatment (Figure 6). When WM852c cells were treated with the combination of ABT-737 and MG-132, blocking Noxa expression by siNOXA significantly decreased total Annexin V^+ cells by almost 50% (from 64.8 ± 1.9 to

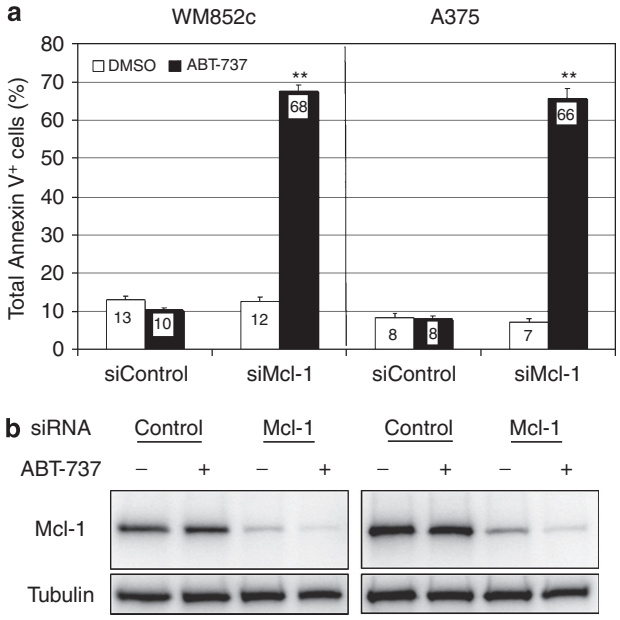

Figure 5. Knocking down Mcl-1 expression increased melanoma cell sensitivity to ABT-737. Melanoma cells were transiently transfected with siRNA targeting human Mcl-1 (siMcl-1) or the nontargeting control pool (siControl). Cells were then cultured for 24 hours followed by a treatment with either DMSO or 3.3 μM ABT-737 for an additional 24 hours. (**a**) Annexin V apoptosis assay. For cells treated with ABT-737, one-way ANOVA demonstrated significantly higher numbers of Annexin V^+ cells for siMcl-1 transfectants compared with siControl transfectants (**: WM852c, $F=1049.1$, $F_{crit}=7.7$, $P=5.4\times10^{-6}$; ##: A375, $F=461.8$, $F_{crit}=7.7$, $P=2.8\times10^{-5}$). Results represent percentages ± SEM for three independent experiments and (**b**) immunoblot.

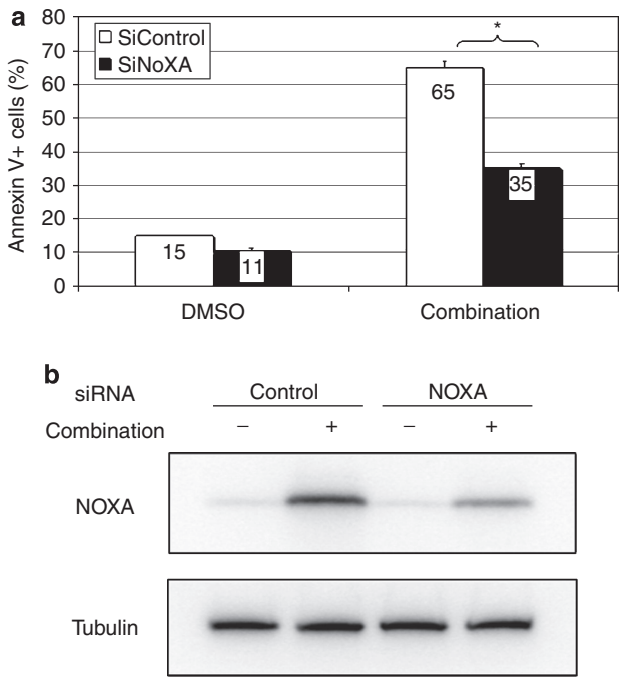

Figure 6. Blocking Noxa induction rescued cells from synergistic killing by the combination of MG-132 and ABT-737. WM852c melanoma cells were transiently transfected with siRNA targeting human Noxa (siNOXA) or the nontargeting control pool (siControl). Cells were then cultured for 30 hours followed by a treatment with either DMSO or the combination of 1.1 μM ABT-737 and 0.18 μM MG-132 for 18 hours. (**a**) Annexin V apoptosis assay. For cells treated with the combination, one-way ANOVA demonstrated significantly lower numbers of Annexin V^+ cells for siNoxa transfectants compared with siControl transfectants (*: ANOVA, $F=171.6$, $F_{crit}=7.7$, $P=0.0002$). Results represent percentages ± SEM for three independent experiments and (**b**) immunoblot.

35.0 ± 1.2%; analyses of variance (ANOVA), $P=0.0002$). In addition, immunoblot analysis indicated that siNOXA inhibited Noxa induction by ~50% under the combination treatment condition (Figure 6b). These results suggest that Noxa is a major mediator of apoptosis induced by the combination drug treatment.

Take together, Figures 5 and 6 suggest that Mcl-1 mediates resistance to ABT-737, and the combination treatment of MG-132 and ABT-737 antagonizes Mcl-1 through increased Noxa expression and caspase-dependent Mcl-1 cleavage. These observations explain the potent synergy observed for the combination treatment.

DISCUSSION

Malignant melanoma is a devastating disease as it metastasizes early and is highly resistant to all conventional treatments. This study investigates whether the strategies that combat anti-apoptotic defenses, such as the use of the small molecule inhibitor ABT-737, might be synergistic with proteasome inhibitors for treating melanoma. By examining multiple parameters of cell viability and apoptosis activation, Figures 1 and 2, Figures S1 and S2 strongly demonstrated that ABT-737 and MG-132 synergistically induce apoptosis in melanoma cells. In addition, active Bak/Bax staining confirmed that co-treatment of ABT-737 and MG-132 induced apoptosis through the intrinsic pathway (Figures 2c and Figure S3). Thus, these data reveal the strong synergy between ABT-737 and MG-132 in killing melanoma cells through mitochondria-mediated apoptosis, and they validate targeting melanoma's anti-apoptotic defenses as a rational molecular approach to therapy.

These results are consistent with a recent report that also showed high efficacy in treating melanoma cells by combining a proteasome inhibitor (bortezomib) with the natural product (−)-gossypol, a different inhibitor of the anti-apoptotic proteins Mcl-1/Bcl-2/Bcl-X_L (Wolter *et al.*, 2007).

It has been shown that the BH3-only pro-apoptotic protein Bad binds tightly to the anti-apoptotic proteins Bcl-2, Bcl-X_L, and Bcl-w, but not to Mcl-1, whereas Noxa bind to Mcl-1 and A1 (Chen *et al.*, 2005; Willis *et al.*, 2005). In accord with their complementary binding, Bad and Noxa together neutralize four major anti-apoptotic proteins and cooperate to induce potent killing (Chen *et al.*, 2005; Willis *et al.*, 2005).

ABT-737 is a mimetic of Bad, as it binds tightly to Bcl-2/Bcl-X_L/Bcl-w and antagonizes their anti-apoptotic function (Oltersdorf *et al.*, 2005). Just as Bad does, ABT-737 has been shown to induce apoptosis synergistically with overexpressed Noxa (van Delft *et al.*, 2006). In addition, high expression of Mcl-1 (Noxa's anti-apoptotic counterpart) is a critical factor for cellular resistance to ABT-737, and resistant cells can be sensitized by various approaches that downregulate, desta-

bilize, or inactivate Mcl-1 (Konopleva *et al.*, 2006; van Delft *et al.*, 2006; Chen *et al.*, 2007a, b). We found that knocking down Mcl-1 expression significantly increased melanoma cell sensitivity to ABT-737 (Figure 5), indicating that Mcl-1 is the main barrier for ABT-737-induced cell death.

In melanoma, proteasome inhibitors such as MG-132 and bortezomib have been reported to induce high levels of Noxa expression and lead to Noxa-mediated apoptosis (Fernandez *et al.*, 2005; Qin *et al.*, 2005). We hypothesized that ABT-737 would be synergistic with proteasome inhibitor such as MG-132 in killing melanoma cells, as ABT-737 blocks the function of Bcl-2, Bcl-X_L, Bcl-w, whereas MG-132 would induce Noxa production, neutralizing the anti-apoptotic function of Mcl-1. Thus, ABT-737 and MG-132 together would inhibit four major anti-apoptotic proteins to synergistically induce apoptosis.

We found that the combination of ABT-737 and MG-132 indeed induced high levels of Noxa expression, similar to MG-132 treatment alone (Figures 3 and Figure S4a). We also demonstrated that the combination induced Noxa-dependent apoptosis (Figure 6), even though Noxa induction alone was not sufficient to induce apoptosis effectively in these cells (see MG-132 treatment alone in Figures 1 and 2). Thus, one of the mechanisms for potent killing synergy between ABT-737 and MG-132 is that the combination acts similarly to overexpressing Bad plus Noxa. As a result, the combination treatment effectively neutralizes all four main anti-apoptotic proteins, therefore promoting cooperation to induce potent killing in melanoma.

Interestingly, proteasome inhibitors also have been reported to induce accumulation of the anti-apoptotic protein Mcl-1 (Fernandez *et al.*, 2005; Qin *et al.*, 2005), and reducing Mcl-1 levels (using siRNA, UV light, or fludarabine) synergizes in causing proteasome-inhibitor-induced cell death in melanoma cells (Fernandez *et al.*, 2005; Qin *et al.*, 2005; Gomez-Bougie *et al.*, 2007). Even partial downregulation of Mcl-1 causes a two-fold increase in proteasome-inhibitor-induced melanoma killing (Qin *et al.*, 2006), and more efficient reduction of Mcl-1 expression dramatically increases and accelerates bortezomib-induced cell death (Wolter *et al.*, 2007).

Although we also observed substantial Mcl-1 accumulation up to at least 24 hours in cells treated with MG-132 alone and at early time points of combination treatment, full length Mcl-1 was quickly degraded in the combination treatment at 24 hours (Figures 3 and Figure S4). This rapid destruction of Mcl-1 might also contribute to potent synergy; likely because ABT-737 plus MG-132 simultaneously triggers Noxa production (by MG-132) and reduces Mcl-1 levels (by ABT-737 plus MG-132).

Rapid degradation of Mcl-1 can be induced by certain cytotoxic triggers, including UV irradiation (Nijhawan *et al.*, 2003; Willis *et al.*, 2005), DNA damage by viral infection (Cuconati *et al.*, 2003) and detachment (Woods *et al.*, 2007). Further, destruction of Mcl-1 is essential for apoptosis induced by these stimuli (Cuconati *et al.*, 2003; Nijhawan *et al.*, 2003; Willis *et al.*, 2005; Woods *et al.*, 2007), and elimination of Mcl-1 may help to ensure irreversible commitment to apoptosis.

In our case, the combination of ABT-737 and MG-132 induced Mcl-1 degradation swiftly in melanoma cells, which may be another reason for the potent killing synergy seen in the combination of these two compounds. This rapid destruction of Mcl-1 might help overcome cellular resistance to both MG-132 and ABT-737. As shown in Figure 4, the wide-spectrum caspase inhibitor zVAD-fmk almost completely blocked Mcl-1 degradation induced by co-treatment with ABT-737 and MG-132. This demonstrates that Mcl-1 degradation in the combination treatment is mainly caspase-dependent, although this might only be a by-product of the apoptotic cascade. However, studies have shown that caspase-dependent Mcl-1 cleavage can be induced by various stimuli, including the proteasome inhibitor bortezomib, for example, in multiple myeloma (Clohessy *et al.*, 2004; Michels *et al.*, 2004; Gomez-Bougie *et al.*, 2007). Further, caspase-dependent Mcl-1 cleavage has been shown not only to abrogate Mcl-1's anti-apoptotic function, but also to generate pro-apoptotic Mcl-1-truncated proteins which enhance the death signal (Clohessy *et al.*, 2004; Michels *et al.*, 2004). Moreover, a recent study demonstrated that cleavage-resistant Mcl-1 potently inhibits mitochondrial-mediated apoptosis (Chen *et al.*, 2007a, b). Thus, we propose that caspase-dependent Mcl-1 cleavage is also a part of a positive feedback loop in our study, which accelerates and synergizes the killing effects of ABT-737 combined with MG-132 in treating melanoma.

Figure 7 summarizes our hypothesis describing the mechanisms of potent synergy between ABT-737 and MG-132 in melanoma. Under normal conditions, cells are kept in check by anti-apoptotic proteins such as Bcl-2, Bcl-X_L, Bcl-w, and Mcl-1 (apoptotic inhibitors in Figure 7). On co-treatment of ABT-737 and MG-132, the Bad mimetic ABT-737 initially blocks the function of Bcl-2, Bcl-X_L, and Bcl-w, whereas MG-132 induces Noxa production, which in turn, neutralizes the anti-apoptotic function of Mcl-1. Thus, ABT-737 and MG-132 together inhibit all four anti-apoptotic proteins, tilting the balance toward Bax/Bak activation and inducing Noxa-dependent apoptosis.

Secondarily, when a slight amount of caspase is activated, Mcl-1 is cleaved. This eliminates the full length Mcl-1

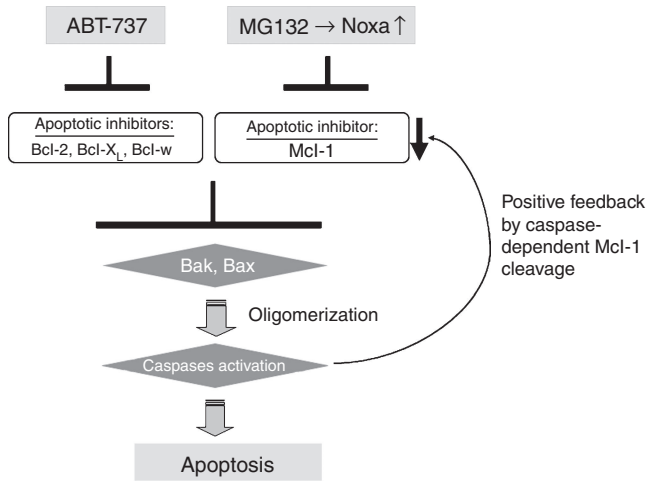

Figure 7. Proposed model for the synergistic mechanism of ABT-737 and MG-132 combination treatment.

(induced by MG-132) quickly, and generates pro-apoptotic Mcl-1 cleavage products that synergize even further in the death signal. Therefore, caspase-dependent Mcl-1 cleavage produces a positive feedback signal for apoptosis induced by the combination of ABT-737 and MG-132.

In conclusion, this study demonstrates that ABT-737 lowers the apoptotic threshold for the proteasome inhibitor MG-132 in melanoma to induce Noxa-dependent mitochondrial-mediated apoptosis very effectively in melanoma cells. These effects are probably due to both drugs' combined ability to neutralize multiple anti-apoptotic proteins and induce caspase-dependent Mcl-1 cleavage to create a positive feedback death signal. These results not only provide scientific basis for further animal and clinical studies to evaluate the efficacy of this combination in treating melanomas, but also implicate the importance of testing additional drug combinations that simultaneously target multiple anti-apoptotic defenses.

MATERIALS AND METHODS

Cell lines and culture conditions

A375, an advanced vertical growth phase human melanoma cell line, was obtained from ATCC (Manassas, VA). WM852c and HS294T, two metastatic human melanoma cell lines, were kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). Cells were maintained in RPMI1640 (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (Gemini Bio-Products Inc., West Sacramento, CA). A375 has a $BRAF^{V600E}$ mutation and no common mutations in NRAS (exon 1 or 2), and WM852c has an $NRAS^{Q61R}$ mutation but no BRAF mutation (exon 11 or 15).

Reagents

MG-132 and z-VAD-FMK were purchased from EMD Biosciences (San Diego, CA) and R&D Systems (Minneapolis, MN), respectively. ABT-737 was kindly provided by Abbott Laboratories (Abbott Park, IL).

Cell titer 96 aqueous one solution cell proliferation assay for quantification of cell viability (MTS assay)

The reagents were obtained from Promega (Madison, WI), and procedures were followed as previously described (Shellman *et al.*, 2005).

Measurement of apoptosis using Annexin V staining

The Annexin V–fluorescein isothiocyanate Apoptosis Detection Kit (BD Biosciences, San Jose, CA) was used according to the manufacturer's protocol. Cells were analyzed by flow cytometry using a Beckman Coulter FC500 with CXP software (Hialeah, FL) in the University of Colorado Cancer Center Flow Cytometry Core.

Immunoblot

Cells, both floating and adherent, were harvested with 1 × Laemmli sample buffer (Bio-Rad, Hercules, CA). Samples were used in the standard western blot analysis protocol as described previously (Ruth *et al.*, 2006). Blots were developed with horseradish peroxidase substrate (West Pico or Femto solutions, Pierce, Rockford, IL) for 5 minutes at room temperature, and analyzed using a Chemi-doc chemiluminescence detector (Bio-Rad). The following antibodies were used at the suggested dilution from the manufacturers: caspase 3, poly (ADP-ribose) polymerase, Bax, and tubulin α/β from Cell Signaling Technology (Danvers, MA), Noxa, anti-actin mouse monoclonal antibody and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin M from EMD Biosciences Inc., Bad, Bcl-x, Mcl-1, and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G from BD Biosciences, Bim from Chemicon (Temecula, CA), Bcl-2 from Dako (Glostrup, Denmark), and horseradish peroxidase-conjugated goat anti-mouse IgG from Jackson Immuno-Research (West Grove, PA).

Measurement of mitochondrial-mediated apoptosis with active Bax/Bak staining

Cells were stained with a protocol modified as previously described (Qin *et al.*, 2006). Cells were detached and fixed in 2% formaldehyde (PolySciences Inc., Warrington, PA) (10 minutes, room temperature), and then washed with FACS buffer (phosphate-buffered saline solution containing 5% fetal bovine serum and 0.02% sodium azide) three times. Cells were permeabilized briefly with 0.03% saponin/FACS buffer before incubated with primary antibody in the same buffer (30 minutes, room temperature). Mouse anti-active-human-Bax antibody (BD Pharmingen, San Jose, CA) at a 1:100 dilution, and mouse anti-active-human-Bak antibody (EMD Biosciences) was used at a 1:250 dilution. Fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) were used in 0.3% saponin/FACS buffer at a 1:750 dilution (30 minutes in the dark, room temperature). FACS buffer was used as the washing solution for three times after incubation with each antibody. Stained cells were analyzed by flow cytometry in the University of Colorado Cancer Center Flow Cytometry Core. Average percent increase in Bak/Bax activated cell was calculated as the percent increase of active Bak^+/Bax^+ cells in treated conditions over the DMSO controls.

RNA interference

ON-TARGETplus SMARTpools of siRNA were purchased from Dharmacon Inc. (Lafayette, CO), targeting human Noxa (PMAIP1, NM_021127), human Mcl-1 (MCL1, NM_182763), and the non-targeting control pool. siRNA was introduced into cells using an Amaxa Nucleofector II device (Amaxa Inc., Gaithersburg, MD). One million cells and 2.5 μM siRNA were used with Amaxa's Reagent V and program X-005 for each reaction.

Cells were nucleofected with indicated siRNA and cultured for indicated amount of time. Then all medium was removed and fresh medium containing indicated compounds for indicated time before the cells were harvested for analysis.

Statistical analysis

Statistical analyses were performed similarly for both Annexin V staining and active Bak/Bax staining. One-way ANOVA were performed to compare group means, and Tukey's *post hoc* tests were used to compare treated samples to DMSO controls.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplemental Results, Material and Methods

Figure S1. Combination of ABT-737 and MG-132 killed melanoma cells synergistically demonstrated by Combination Index values.

Figure S2. Combination of ABT-737 and MG-132 killed melanoma cells synergistically as analyzed by EB/AO staining.

Figure S3. The effects of combination treatment with ABT-737 and MG-132 on Bcl-2 family member protein expression.

Figure S4. The effects of combination treatment with ABT-737 and MG-132 on Noxa and Mcl-1 protein expression.

Figure S5. Caspase inhibitor zVAD-fmk blocked downregulation of Mcl-1 protein expression induced by ABT-737 and MG-132 combination treatment in WM852c cells.

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